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13. ABSTRACT (Maximum 200) Our goal was to design, synthesize and screen novel organic chemicals designed to inhibit all or several of the known botulinum toxin (BoNT)-metalloproteases. We have succeeded in demonstrating the feasibility of our approach to the design of botulinum inhibitors based on using the weak activity of captopril as a lead compound. We report the first prototype compounds that exceed our captopril lead compound by at least an order of magnitude in inhibitory properties. Such activity could be substantially enhanced by the resolution of the stereo enteriomer compounds into the optical isomer possessing the biological activity. We also confirm that we can synthesize the compounds on solid phase and have the capability to create large libraries by applying this approach. Finally, we have evidence that more potent, selective inhibitors are possible. Innovations in combinatorial chemistry applied to structural leads discovered in Phase I will form the rationale for developing tight-binding inhibitors of BoNT-metalloproteases. Additionally, we have in hand the requisite light chain derivatives to accomplish high throughput screening of our synthetic libraries.				
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FOREWORD

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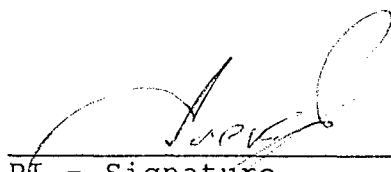
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I. Introduction

Our research was in response to Topic A96-138, "Development of Pharmacologic Antagonists for Botulinum Toxin." The botulinum and tetanus toxin zinc metalloproteases identified by Montecucco, Schiavo and their collaborators, cleave single amide bonds in VAMP/synaptobrevin, SNAP-25 and syntaxin (1). These metalloproteases are formed from the parent toxin itself by limited proteolysis inside endocytotic vesicles and then transported into the cytosol where they selectively cleave vesicle associated proteins. This process damages or destroys cholinergic action, especially at the neuromuscular junction, and causes the paralysis characteristic of botulinum toxin poisoning.

Our goal was to design, synthesize and screen novel organic chemicals designed to inhibit all or several of the known Botulinum toxin (BoNT) metalloproteases. The Phase I aims of our research are listed below.

Table I Research Specific Objectives

1. Design novel zinc binding ligands for incorporation into peptide substrate sequences
2. Modify known zinc binding ligands to mimic BoNT substrate sequences
3. Develop solution chemical methods for synthesizing analogs of captopril in a combinatorial chemical approach
4. Extend the solution methods developed in part #3 to develop a synthesis of small libraries of peptide-derived and non-peptide derived BoNT-metalloprotease inhibitors on solid phase Tentagel resins
5. Assay all synthetic compounds for inhibition of cleavage of recombinant substrates by recombinant BoNT/B and -E metalloprotease

II. Research Conducted

A. Synthesis of Zinc Chelators Related to Captopril and Enalapril.

In our original application, we showed that captopril is a weak but measurable inhibitor of two BoNT proteases. In our hands, of the three inhibitors shown below only captopril and enalapril have demonstrable inhibitory activity:

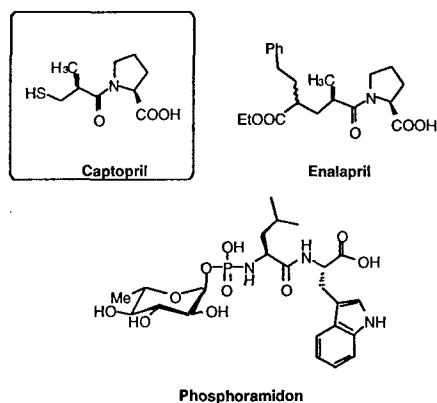


Fig 1. Known Inhibitors of BoNT Proteases

Thus, the sulfhydryl group is probably interacting with the zinc in the active site of the BoNT proteases. We proposed to synthesize and screen numerous analogs of these two compounds, since captopril is a known orally active inhibitor of angiotensin-converting enzyme (ACE) and possess the pharmacodynamic properties needed to penetrate multiple biological membranes in humans. Thus, captopril represents a very important starting point for the development of cell-permeable BoNT-protease inhibitors; it is low molecular weight, has excellent oral bioavailability (about 40%) in humans, and low toxicity to humans.

In Phase I of this research, we proposed to develop the chemistry in solution to prepare a series of Captopril analogs that show improved binding to BoNT metalloproteases in order to proceed to Phase II solid phase synthesis. We have achieved this goal. Our work has established the chemistry needed to synthesize libraries of captopril analogs by combinatorial chemical methods that will be screened with the recombinant target proteins to identify tight-binding inhibitors of BoNT-proteases.

The chemistry follows that described by Gallop and co-workers (2) for preparation of substituted prolines, but is enhanced in that novel structure-activity relationships have been obtained. Figure 2 shows the synthesis of imines, which are reacted with olefins to form pyrroles (Fig 3). These are acylated with mercaptopropionic acid derivatives to generate the S-acyl form (Figure 4); these are deprotected to liberate the active form of the inhibitor in the assay.

Figure 2.

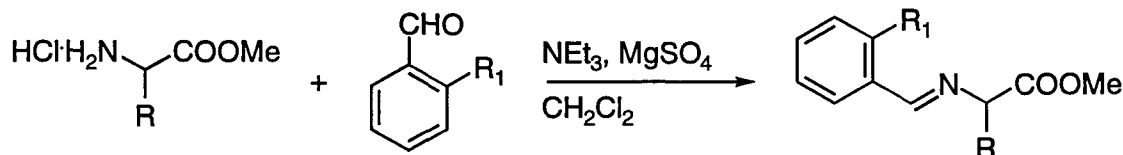


Figure 3.

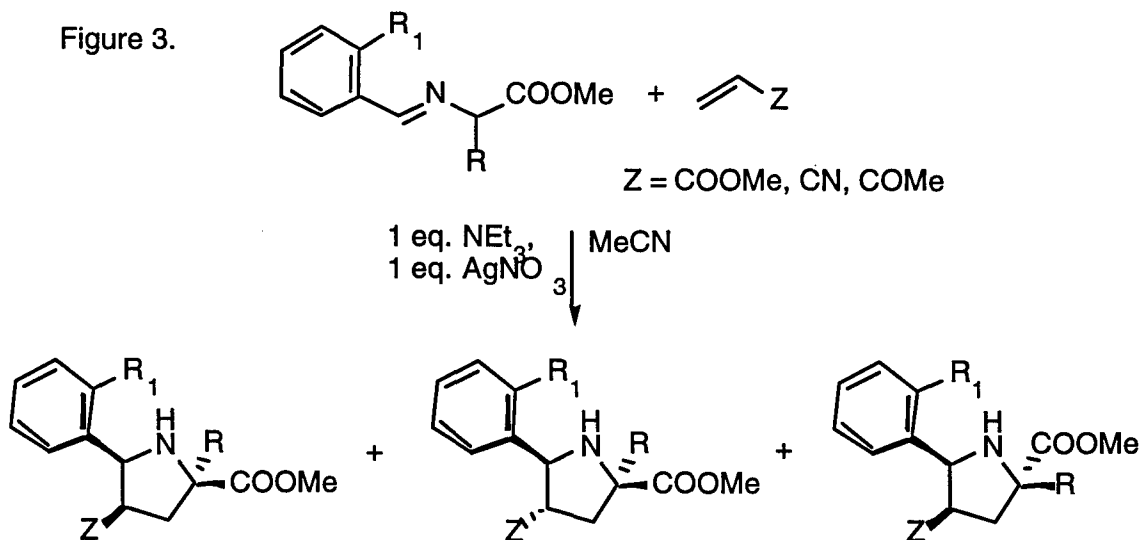
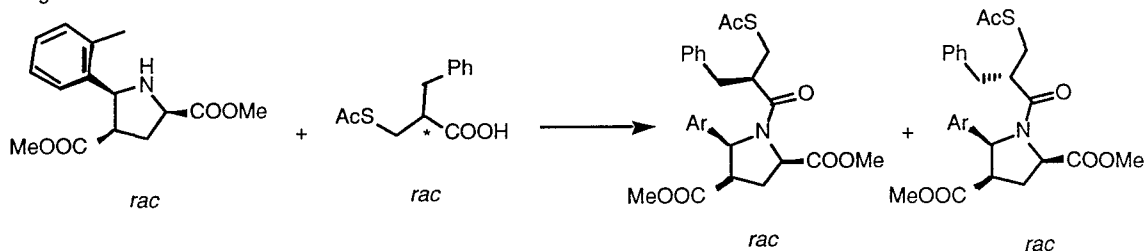


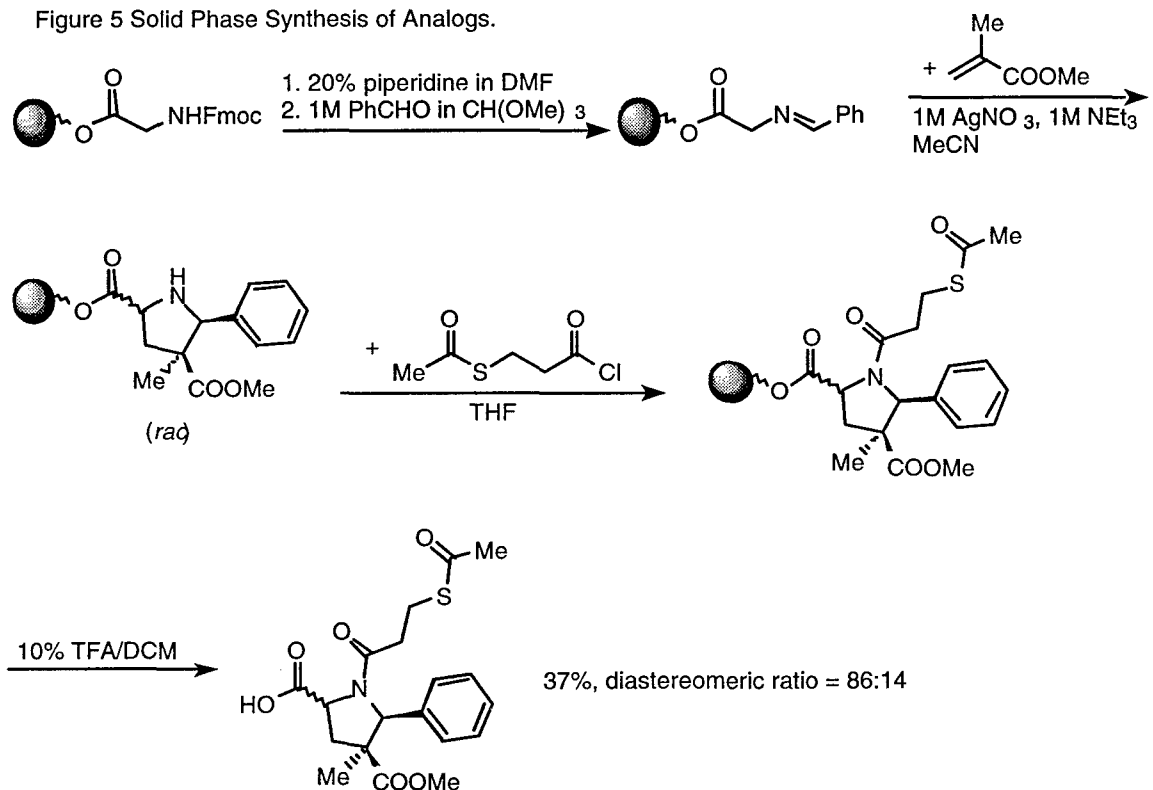
Figure 4.



B. *Modify known zinc binding ligands to mimic BoNT substrate sequence.* In early phases of this research, we prepared a limited number of small analogs of substrate to explore whether inhibitor activity might be found, as was proposed for Objective I in the Phase I proposal. These compounds were not active in our assays, and this approach was soon dropped when the captpril-based approach proved successful.

C. *Extend the solution methods developed in Section A to develop a synthesis of small libraries of peptide-derived and non-peptide derived BoNT-metalloprotease inhibitors on solid phase resins*

Figure 5 Solid Phase Synthesis of Analogs.



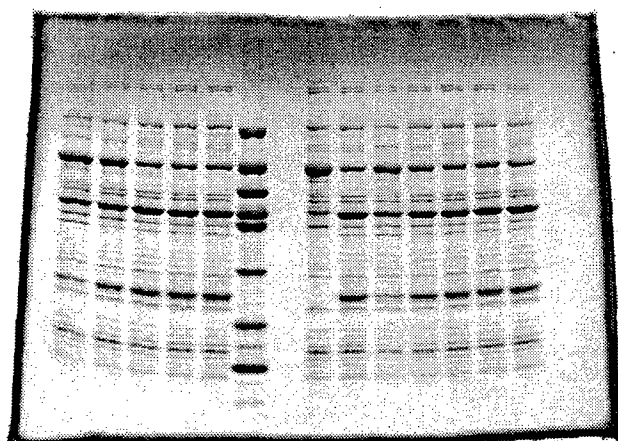
The solution phase chemistry used to synthesize the compounds (refer to Section A, Figures 2-4) has been extended to solid phase methods thereby enabling great enhanced productivity for the synthesis of combinatorial libraries for Phase II (see Figure 6). We will need to synthesize a large number (> 10,000) of new inhibitors by

combinatorial methods to determine structure-activity relationships and identify tight binding inhibitors. As a result of our work in Phase I we now routinely make these on solid phase.

D. Assay all synthetic compounds for inhibition of cleavage of recombinant substrates by recombinant BoNT metalloprotease

Evaluation of a Lead Compound Screening System — In our Phase I proposal, we described a system to screen compounds for inhibitory activity. This is based on cleavage of a SNAP25-RAP hybrid protein by recombinant light chain of BoNT/E (See Fig. 6 below). In a preliminary experiment, we demonstrated that captopril, a known metalloprotease inhibitor of ACE, inhibited botulinum toxin metalloprotease. We also discovered that enalapril, an ACE metalloprotease inhibitor with hitherto unknown inhibitory activity toward the botulinum toxin, also inhibits processing of SNAP-25 substrate by BoNT/E-protease.

Figure 6 illustrates our assay. Recombinant substrate (lane 8; 64.6 kDa) is processed by BoNT/E-protease to two new bands of apparent mass of 43.4 and 21.2 kDa. Cleavage is inhibited by two metalloprotease inhibitors. In lane 1, 10 mM captopril clearly inhibits buildup of the 43 and 21 kDa fragments relative to controls; in lane 10, 10 mM of enalapril inhibits buildup of the same fragments. In both cases, the 64 kDa band persists longer in the presence of the inhibitors. Thus, captopril and enalapril, two orally active drugs, inhibit BoNT-protease cleavage of SNAP-25.



Lane 1 2 3 4 5 6 7 8 9 10 11 12 13 14

Fig. 6. Cleavage of SNAP25-RAP hybrid with recombinant light chain of BoNT/E and inhibition of this cleavage by captopril and enalapril.

Enriched *E. coli* extracts containing SNAP25-RAP hybrid in the 10 mM TrisHCl, 1mM ZnSO₄, pH 6.8 were incubated at 37°C for 1 hour alone (lane 8), with recombinant light chain of BoNT/E (lane 9) or with recombinant light chain of BoNT/E and different concentrations of captopril (lanes 1-5) enalapril (lanes 10-14). Concentrations of inhibitors used were 10mM (lanes 1 and 10), 1mM (lanes 2 and 11), 100 μM (lanes 3 and 12), 10 μM (lanes 4 and 13) and 1 μM (lanes 5 and 14). Products of proteolysis were analyzed using 4-16% SDS-PAGE followed by staining with Coomassie Blue. Lane 6 is Promega's mid-range molecular weight markers. Lane 7 is light chain of BoNT/E in the amount used in each reaction

In addition to the SNAP25-RAP hybrid, we have constructed a hybrid between cytosolic portion of rat synaptobrevin 2 and RAP. Similarly to SNAP25-RAP, this hybrid was efficiently produced in *E. coli* and was found to be stable. Also this hybrid was found to be an efficient substrate for recombinantly produced light chain of BoNT/B.

Our experience with SNAP25-RAP and synaptobrevin 2-RAP hybrids convinced us that both SNAP25 and synaptobrevin 2 could be fused with other polypeptides and such fusions do not interfere with the ability of these proteins to serve as substrates for enzymatic domains of BoNTs. We believe that such a 'hybrid' approach dramatically improves the procedure for screening of potential inhibitors of BoNTs. The simple procedure we used during Phase I was proven to be reliable for screening small quantities of potential inhibitors by direct monitoring of disappearance of substrates and appearance of products by means of SDS-PAGE. Indeed in the case of fusion proteins difference between molecular weights of substrates and products was more profound than the same difference in the case of SNAP25 and synaptobrevin 2. As a result experiments exploiting fusion proteins were convenient to handle and results were easy to analyze (compare Figs. 6 and 7).

The complication of using synaptobrevin 2-RAP as substrate for BoNT/B was associated with the fact that this hybrid protein had a tendency to aggregate. To keep this substrate soluble we had to add heparin to the solution. Potentially the presence of heparin in the reaction mixture could interfere with the reaction. Therefore we have analyzed cleavage of synaptobrevin 2 in the presence or absence of heparin. For this purpose we have constructed a gene encoding the cytosolic part of synaptobrevin 2. Analysis revealed that product of this gene is stable in *E. coli* and is produced as a soluble protein. However, the level of production was less than that for synaptobrevin-RAP. Subsequently, we have developed procedures for isolating and purifying this protein from *E. coli* extracts. Unlike synaptobrevin 2-RAP this protein does not have tendency to aggregate and does not require addition of heparin to stay in the solution. Using this protein we were able to demonstrate that at a concentration used earlier in our assay system, heparin does not have an effect on the results of proteolytic cleavage (Fig. 7).

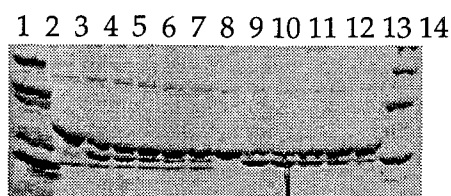


Fig. 7. Effect of heparin on cleavage of synaptobrevin 2 with light chain of BoNT/B.

Synaptobrevin alone (2 and 8) or with decreasing concentrations of BoNT/B (3-7 and 9-13) has been incubated for 2 hours at 37°C in buffer (10 mM TrisHCl pH 6.8, 1 mM ZnSO₄) either with 1mg/ml of heparin (6-13) or without (2-7). Then samples were separated on 4-20% SDS-PAGE and stained with Coomassie. Lanes 1 and 14 correspond to molecular weight standards

We would like to exploit the fact that fusions between SNAP25 and synaptobrevin 2 with other proteins are recognized and cleaved by BoNTs. Particularly we believe that SNAP25 or the cytosolic portion of synaptobrevin 2 fused on their N-terminal with a peptide that is biotinylated in *E. coli* (1), and on their C-terminal fused with such easy detectable and quantifiable proteins as Green Fluorescent Protein (GFP) or phosphatases could be used to develop a robust procedure for measuring BoNTs activities and activities of BoNTs inhibitors. During Phase I, we attempted to 'tag' synaptobrevin 2 with GFP. Our attempts to produce such a hybrid in *E. coli* have failed. We believe that for some as yet unknown reason, fusion of GFP with other proteins destabilizes these proteins in *E. coli*. The fact that our attempts to produce fusions between BoNT/B and GFP have failed seems to support this conclusion. Despite the failure to label BoNT substrates with GFP we believe that use of labeled derivatives of SNAP25 and synaptobrevin 2 could be viable approaches to detect and quantify activities of BoNTs. Therefore during Phase II we intend to proceed further with construction of such derivatives, this time using phosphatase.

Additional Work on the Screening Assay — We were able to find conditions that allow efficient production of fragments of clostridial neurotoxins in *E. coli*. Using these conditions, we cloned and expressed light chains of botulinum neurotoxins serotypes B, C and E as well as light chain of tetanus toxin. Dr. Zdanovsky's group purchased and installed a Pharmacia FPLC system to enable the large scale preparation of the required protein substrates; a critical element in our assay regime as well as planned Phase II activities. During the second month of our research, we established an optimal procedure for large scale renaturation and purification of the recombinant enzymatic domain BoNT/B for use in our assay system. Additionally we recovered an enzymatically active fragment of botulinum neurotoxin E. With the help of the FPLC system we plan to scale up this process during Phase II. We also plan to establish similar processes for enzymatic domains of BoNT/C and tetanus toxin. Using recombinant enzymatic domains of BoNT/B and synaptobrevin 2-RAP, we were able to establish system for screening of potential inhibitors of BoNTs enzymatic activities (see Fig. 8 on the following page).

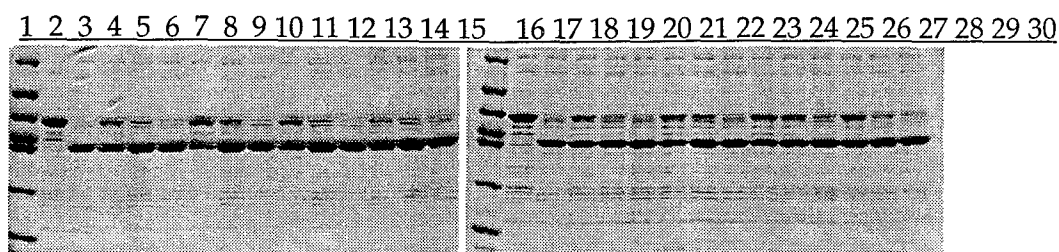


Fig. 8.
Screening of Lead Compounds in the BoNT/B - synaptobrevin compounds -RAP system.

Synaptobrevin-RAP alone (2 and 17), with BoNT/B (3 and 18) or with BoNT/B and 1mM (4, 7, 10, 13, 19, 22, 25 and 28), 400 •M (5, 8, 11, 14, 20, 23, 26 and 29) or 100 •M (6, 9, 12, 15, 21, 24, 27 and 30) of potential competitor were incubated for 2 hours at 37°C in buffer containing 10 mM TrisHCl pH 6.8, 1 mM ZnSO₄ and 1mg/ml of heparin. Then samples were separated on 4-20% SDS-PAGE and stained with Coomassie. Lanes 1 and 16 correspond to molecular weigh standards. Inhibitors used: (rac)-CB88a (4-6), (rac)-CB62 (7-9), (rac)-CB92 (10-12), (rac)-CB64 (13-15), CB114b(19-21), (rac)-CB115 (22-24), (rac)-CB117a (25-27)) and (rac)-CB117b (28-30).

Two biphenylether inhibitors provided by Dr. Rich as potential inhibitors of BoNT proteases were tested using SNAP25-RAP hybrid and light chain of botulinum neurotoxin serotype E. Compound #1 shown below (Fig. 9) had a detectable inhibitory at 10 mM concentration. This is the first discovery of a K-13 like structure to inhibit a BoNT protease and also our first inhibitor of BoNT/E protease.

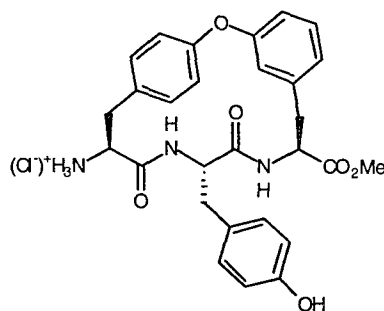


Fig. 9.

Work Directed at a High Throughput Screening System — We have a long term goal of eliminating the tedium and time consuming gel electrophoretic method described above (Figure 6 and 8) and used during Phase I for screening of potential inhibitors. We have focused our efforts on developing a rapid specific method for detecting inhibitors of BoNT protease activity. We believe that such molecules should tightly bind with BoNTs catalytic center. Therefore we have concentrated our work on construction of labeled enzymatic domains of BoNTs. Such hybrids could be used to label in mixtures of synthetic beads those containing on their surface individual, potent inhibitors. Beads labeled with easily identifiable enzymatic domains of BoNTs can be isolated manually

after detection under the microscope, or by an automated process. Our approach has been to create hybrid molecules that encode enzymatic domains of BoNTs fused with GFP. As was mentioned, we found that GFP destabilizes this protein in *E. coli*. Therefore in Phase II of this proposal, in addition to stabilizing BoNT-GFP fusions, we plan to fuse enzymatic domains of BoNTs with peptide domains that are biotinylated in *E. coli* and use these proteins in conjunction with rhodamine labeled avidin to identify the synthesized inhibitors that bind to the BoNT protease domain.

III. Research Findings

Figures 10 to 12 on the following pages show the analogs synthesized. These analogs are sorted into most active, intermediate and weakly active analogs according to our protease inhibition screening method. For comparison, note that captopril is one of the weakest inhibitors in the lot. Several new compounds are active at concentrations 10-fold lower than captopril and the optimal combination has not been determined. E.g. CB62 is about 300 μ M as the racemate and is about 10-fold better than captopril and CB117 appears slightly stronger. These compounds have no activity against BoNT/E, but as noted earlier, the K13 derived inhibitor in Fig 9 does inhibit this enzyme.

Fig. 10 BoNT/B Protease Inhibitors Synthesized. (Assayed as free sulfhydryl).
Most Active Inhibitory Compounds (Active at 1- 0.1 mM)

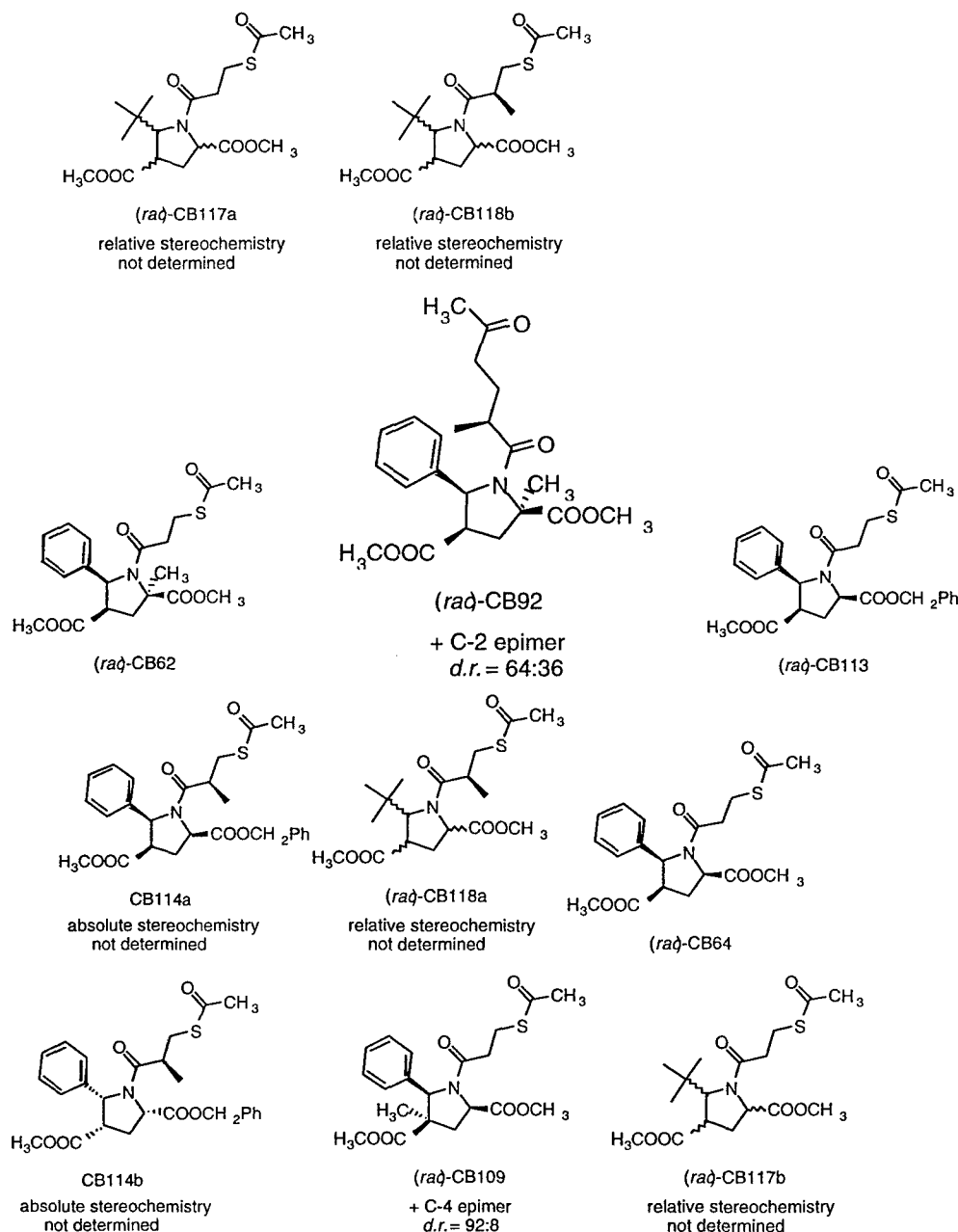


Fig. 11

Intermediate Inhibitory Compounds (Active at > 1 - 5 mM)

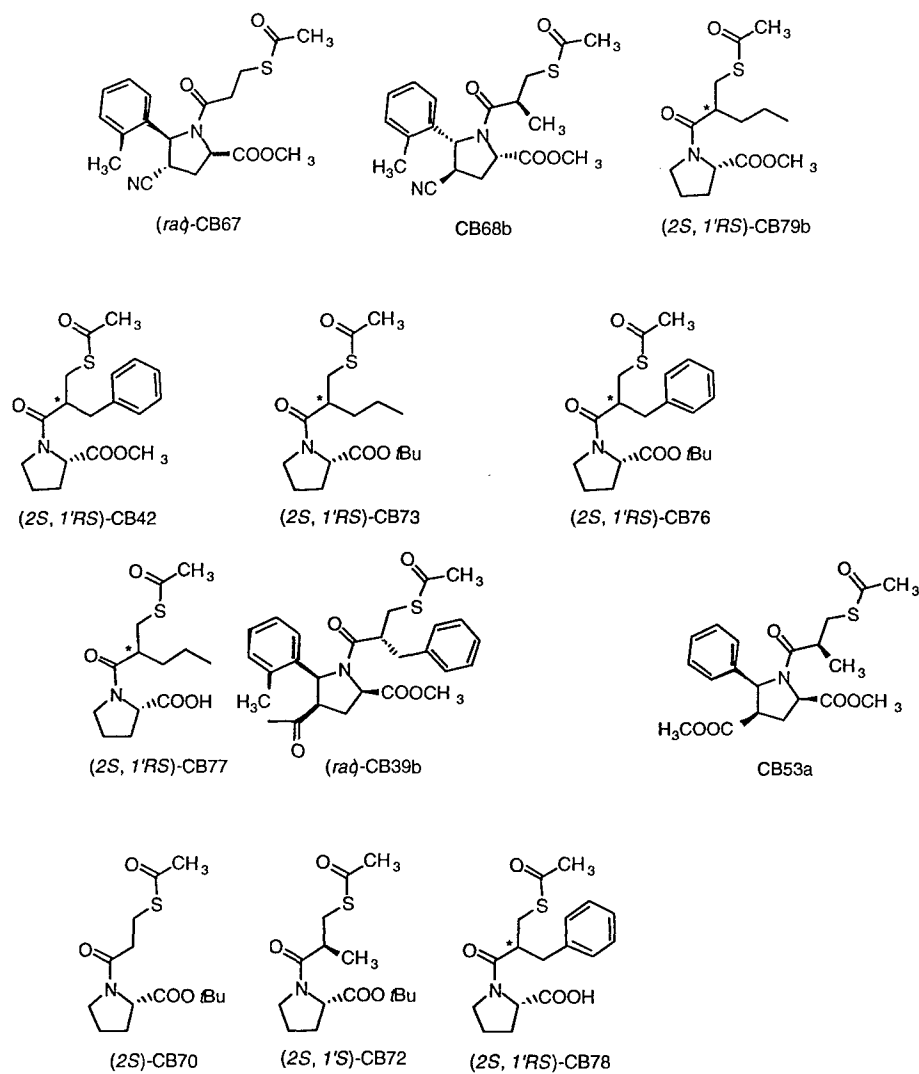
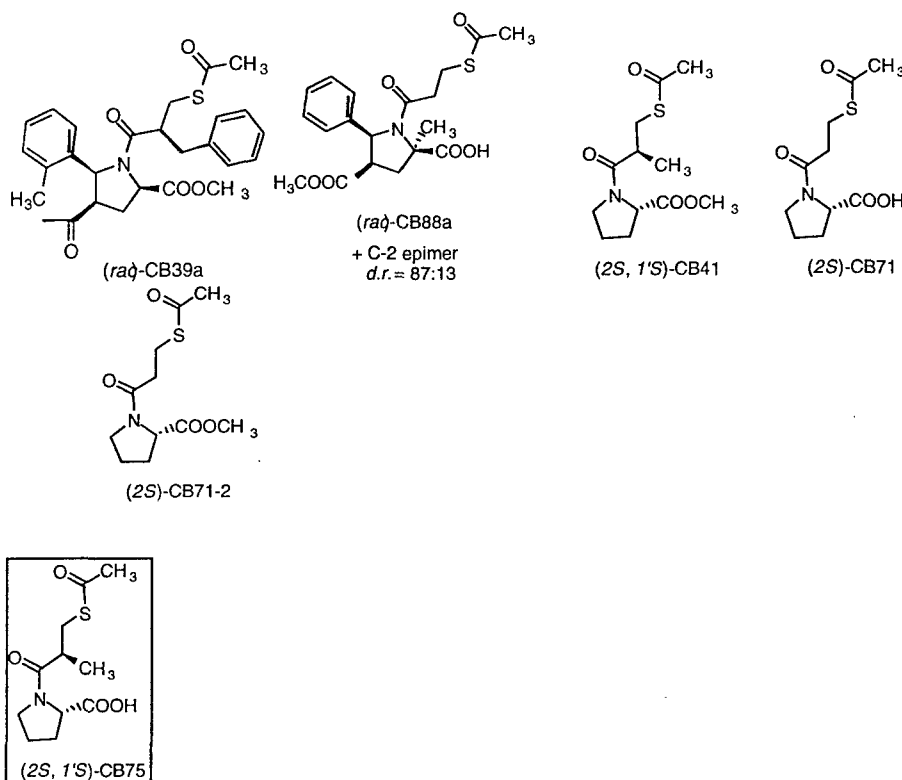


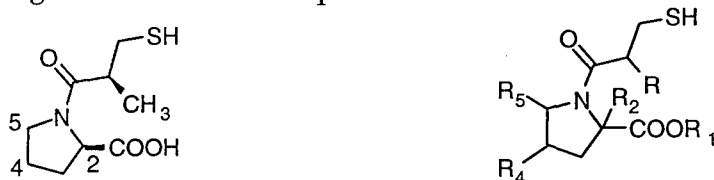
Fig. 12

Weak Inhibitory Compounds (Active at > 5mM)



The structure-activity data for BoNT/B show the following pattern. We have identified absolute differences between ACE inhibitors and BoNT inhibitors (Fig. 13). The most important chemical difference is that the proline carboxyl group prefers to be an ester (or at least not an acid), which is great since it enables us to synthesize these on the resin and assay the analogs while they are still bound to the resin. As stated in the summary, we will need to make many new inhibitors by combinatorial methods to determine structure-activity relationships and identify tight binding inhibitors. We routinely make these on solid phase now.

Figure 13: Structural Requirements to Inhibit ACE and BoNT/B Protease.



ACE Inhibitors:

- needs free carboxyl group
- no α -substituent at C2
- tolerates aromatics/aliphatics at C5
- modest changes at C4

BoTN-Protease Inhibitors:

- carboxyl esters are active
- α -substituent at C2 is active
- likes aliphatics at C5
- modest changes at C3

IV. Conclusions

We have succeeded in demonstrating the feasibility of our approach to the design of botulinum inhibitors based on using the weak activity of captopril as a lead compound. We report the first prototype compounds (see Figure 10) that exceed our captopril lead compound by at least an order of magnitude in inhibitory properties. Such activity could be substantially enhanced by the resolution of the stereo enteriomeric compounds into the optical isomer possessing the biological activity. We also confirm that we can synthesize the compounds on solid phase and have the capability to create large libraries by applying this approach. Additionally, we have in hand the requisite light chain derivatives to accomplish high throughput screening of our synthetic libraries. Finally, we have evidence that more potent, selective inhibitors are possible. In a worse case scenario, we could use a "cocktail" therapeutic approach to inhibit all BoNT proteases by two or more inhibitors, an acceptable therapy at the present time as a result of the protease inhibitor cocktails now in use to treat AIDS, and a feasible goal since we have a lead compound that inhibits BoNT/E as well as the leads for inhibiting BoNT/B.

To further satisfy our Phase I commitment, we have delivered one promising compound from our biological screen to Dr. Michael Adler, Ph.D., Neurotoxicology Branch USA-MRICD for evaluation. Additional compounds can be sent upon request.

V. References to the Literature

1. Schiavo, D., Shone, O. R., Alexander, F. C. G., Montecucco, C. *J. Biol. Chem.* 268, 11516 (1993). See also: Montecucco and Schiavo "Tetanus and botulism neurotoxins: a new group of zinc proteases" in *TIBS* 18, 324 (1993) and Schiavo, G. Poulain, B., Benenati, F., DasGupta, B., and Montecucco, C., (1993) *Trends in Microbiol* 1, (5), 170-4.
2. Murphy, M. M., Schullek, J. R.; Gordon, E. M., and M. A. Gallop, M. A.. Combinatorial Organic Synthesis of Highly Functionalized Pyrrolidines: Identification of a Potent Angiotensin Converting Enzyme Inhibitor from a Mercaptoacyl Proline Library. *J. Am. Chem. Soc.* 1994, 117, 7029-7030.

VI. List of Personnel Receiving Pay from this Research

a) Promega Corporation:

Dr. Alexey G. Zdanovsky, Ph.D.	Principal Investigator
Natalia Karasina, M.S.	Research Assistant

b) Prof. Rich's Laboratory:

Dr. Christiane Boie, Ph.D.	Research Associate
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